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Carmine-Propionic Acid Stain for Elucidation of Fine Cellular Structure in Nematodes

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ABSTRACT: Carmine-propionic acid staining was found to enhance the elucidation of cellular membranes in the esophagus and genital tract and the structure of sperm in strongylate nematodes. This regressive staining technique may also have general utility for differentiation of cellular membranes and nuclear structure in other taxa of parasitic helminths.

Currently there are few staining techniques that have been developed for the study of internal structures in zooparasitic nematodes (Pritchard and Kruse, 1982). Previous research has concentrated on histopathology and detection of free-living and plant-parasitic forms (Daykin and Hussey, 1985; Hooper, 1986). Among these latter groups, stains have also been used in numerous morphological and ontogenetic studies of the excretory and reproductive systems, esophageal structure (e.g., Hirschmann and Triantaphyllou, 1967; Günther, 1973; Rodríguez-Kábana and King, 1977; Permachandran et al., 1988; Aumann, 1994), the peripheral nervous system (Croll and Maggenti, 1968), and oogenesis (Triantaphyllou, 1981).

Among zooparasitic nematodes, stains or staining agents such as iodine have often been used to facilitate the recovery of specimens from intestinal contents at necropsy. Vital stains, including rose bengal or methyl blue, have been used to nonspecifically enhance the contrast of internal characters. Giemsa and acid-fuchsin and other specific stains have been used to elucidate the internal structure of the bursa, genital cone, and spicules in temporary and permanently mounted specimens of some strongylate nematodes (Stringfellow, 1969, 1971). Histochemical staining has also been widely applied in elucidating biochemical activity in nematode tissue (Maki and Yanagisawa, 1980; Fry and Beesley, 1985). However, specimens are most often studied as wholemounts in a standard clearing agent such as glycerine, lactophenol, or phenol–alcohol (Pritchard and Kruse, 1982). Although these procedures allow the examination of cuticular characters and enhance our ability to view structures internal to the cuticle, the details of cellular structure are not clearly discernible.

We report herein a new method for regressive staining of parasitic nematodes. A carmine-propionic acid stain can be used to characterize the structure and distribution of cellular nuclei (particularly the nucleoli), cellular membranes, and tubules (especially in the esophagus, intestine, and reproductive tract), and in comparative morphology of sperm. Methods for fixation and staining are presented below.

The following reagents are required: absolute methanol, 70% ethanol, propionic acid (concentrated), glacial acetic acid, and carmine. The fixative is a solution of 3 parts methanol : 1 part propionic acid (by volume); use of other standard fixatives such as 10% formalin or preservatives such as 70% ethanol is not as effective with this staining procedure. The stain consists of 3.0 g of carmine, 50 ml propionic acid, and 50 ml of distilled water to yield a 3% solution of carmine in 50% propionic acid. Preparation of the stock solution, under an exhaust hood involves the dissolving of carmine in 100 ml of 50% propionic acid held near boiling in a water bath at 100 C for 3 hr, followed by cooling and filtration. Specimens are destained in a solution of equal parts 50% acetic acid and 70% ethanol. Specimens may then be mounted.

Nematodes should be collected while alive. The procedure for fixation and staining is as follows: (1) entire specimens or dissected organ systems to be stained and examined require fixation for a minimum of 2 hr; (2) staining of whole nematodes or dissected organs is achieved by placing material in undiluted stock solution for 4, 12, or 24 hr depending on the size of the specimen; staining times can be reduced by 1–2 hr at 40–50 C; (3) destaining occurs in the acetic acid solution, with stain being removed sequentially while the specimen is observed under a dissecting microscope; optimal staining is similar to the results obtained with other carmine stains (Pritchard and Kruse, 1982); (4) destaining is terminated when specimens are transferred to nonacidified 70% ethanol; (5) specimens are mounted in standard media including glycerine, lactophenol, phenol–alcohol, or polyvinyl alcohol (PVA)–lactophenol. For the latter, clearing may be speeded by using a slide warmer for several hours (Pritchard and Kruse, 1982).

The use of this staining method for differentiation of structure in nematodes is apparent in representative specimens and organ systems of 2 species of strongylate nematodes (Figs. 1–5). Carmine-propionic acid stain enhances and elucidates cellular margins and has an affinity for the nucleoli, which enables the study of the distribution of cellular nuclei as shown in *Metastrongylus apri* (Gmelin, 1790) (Figs. 1–4). Affinity for nuclear components also makes this stain useful for study of sperm morphology as shown in *Dictyocaulus filaria* (Rudolphi, 1809) (Fig. 5). This stain may also have a general utility for studying fine cellular detail or membranes in other parasitic helminths. Representative specimens were deposited as vouchers in the U.S. National Parasite Collection, USDA, Beltsville, Maryland (USNPC nos. 84894 and 84895).

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FIGURES 1–5. Carmine-propionic acid staining for differentiation of cellular structure in *Metastrongylus apri* (Figs. 1–4) and *Dictyocaulus filaria* (Fig. 5). **1,2.** Ovejectors, showing structure of vagina and sphincters, note cellular margins (arrows) (Fig. 1, scale = 100 μm); sphincters and adjacent uterine limbs, arrows indicate cellular margins (Fig. 2, scale = 50 μm). **3.** Esophagus and distribution of esophageal tubules (arrows) and nuclei (scale = 100 μm). **4.** Distribution and structure of nuclei in intestine adjacent to esophagus (scale = 25 μm). **5.** Sperm in the seminal vesicle of a male prior to activation, note conical shape and dense filopodia on body (open arrow) and darkly staining acrosomes (pointers) (scale = 15 μm).

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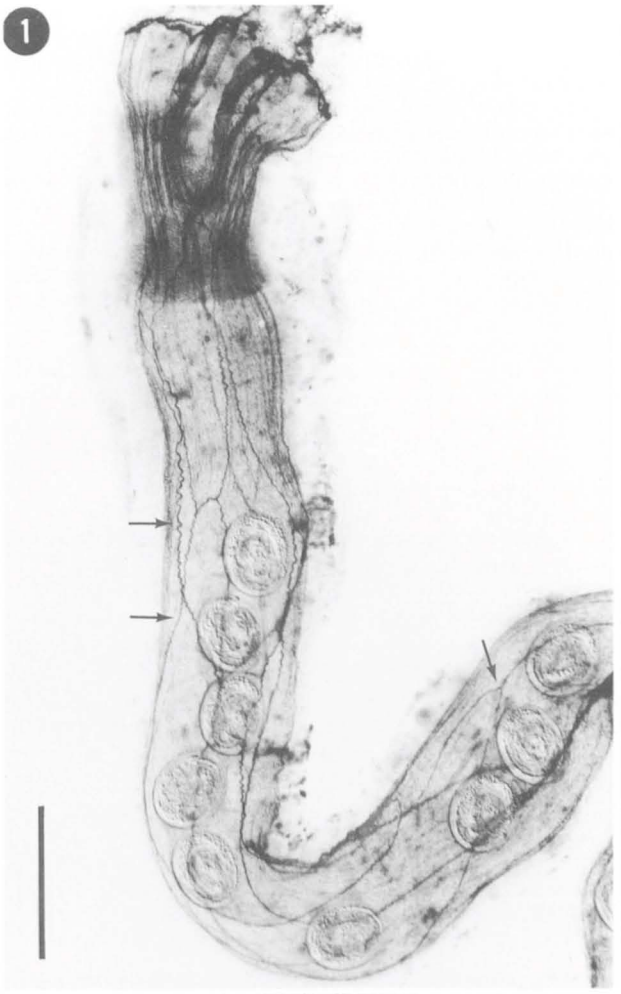

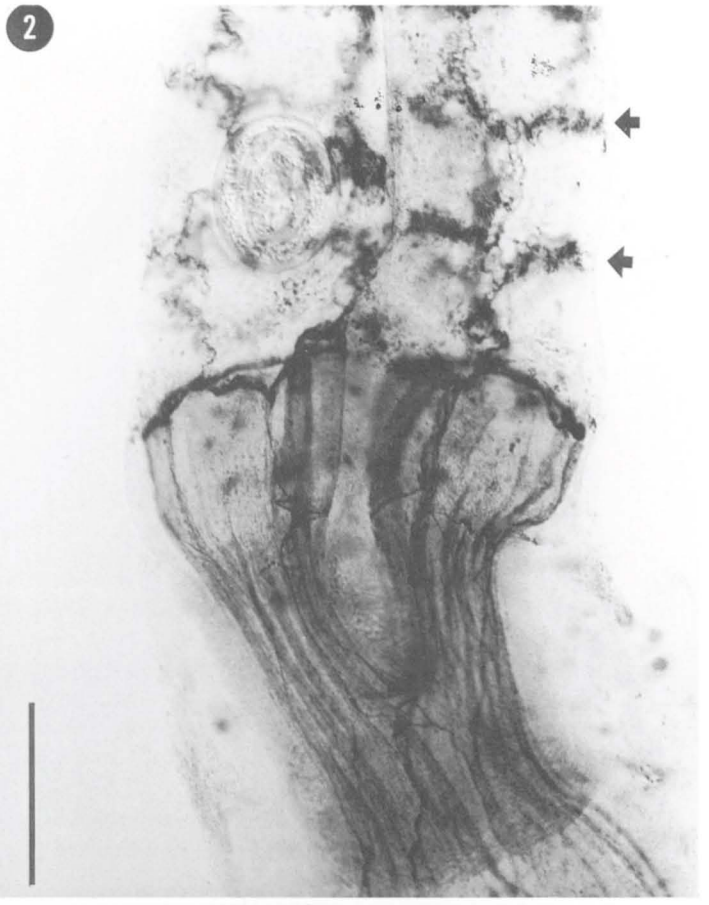

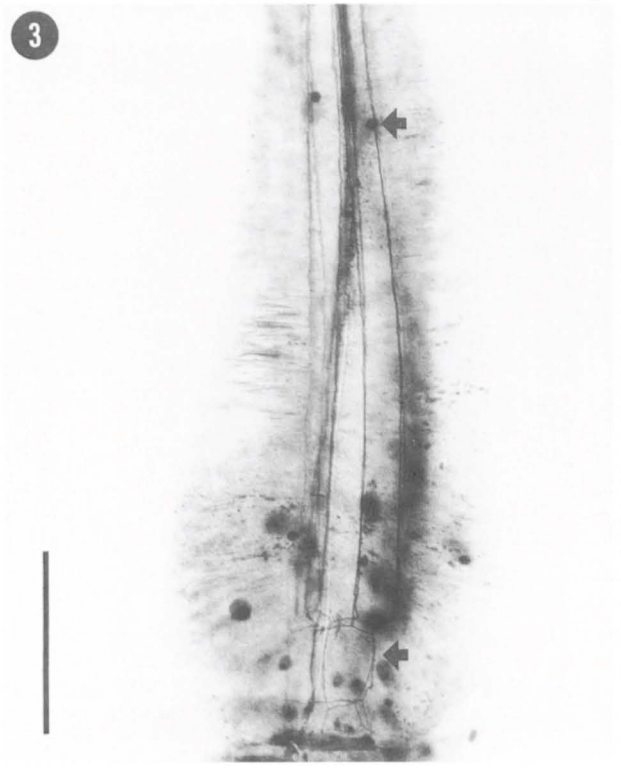

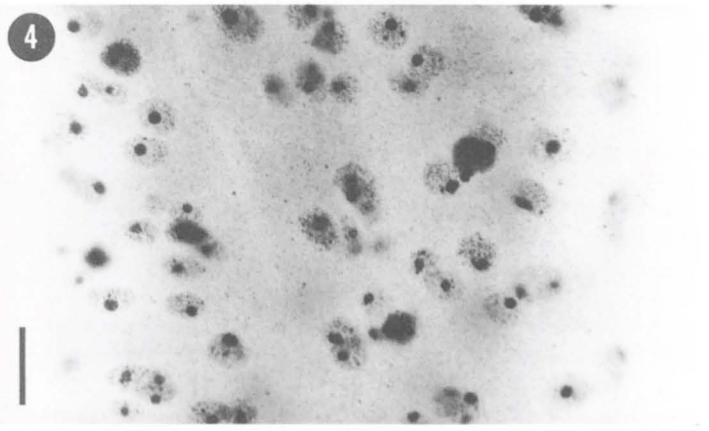

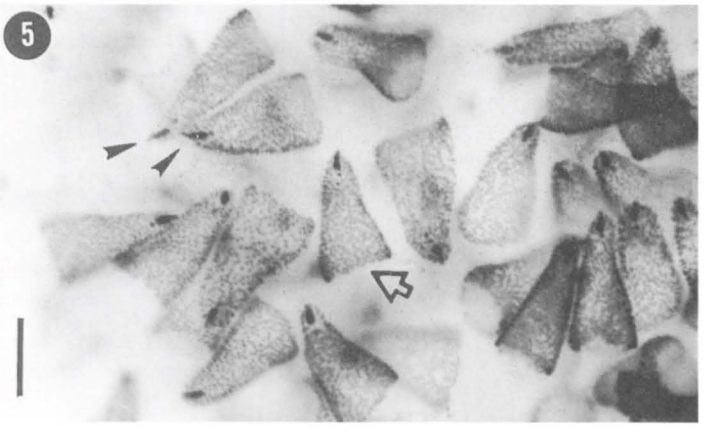

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